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(57) Abstract

The present invention demonstrates that bone morphogenetic proteins (BMPs) are essential for both osteoblast and osteoclast differentiation in bone marrow cell cultures. Furthermore, the present invention demonstrates that noggin, a natural antagonist of BMP action, is able to control and coordinate the continuous supply of osteoblasts and osteoclasts needed for the remodeling of the adult skeleton. Therefore, the present invention is directed towards the use of noggin, or compounds that interfere with noggin, as therapeutic agents in the regulation of osteoblastogenesis and osteoclastogenesis, thereby controlling bone remodeling in the adult skeleton.

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NOGGIN AND ANTAGONISTS OF BONE MORPHOGENETIC PROTEINS TO SUPPRESS PATHOLOGIC BONE RESORPTION

BACKGROUND OF THE INVENTION

Federal Funding Legend

This invention was produced in part using funds obtained through grants P01AG/AR13918 and R01AR43003 from the National Institute of Health. Consequently, the federal government has certain rights in this invention.

Field of the Invention

The present invention relates generally to bone physiology and bone remodeling. More specifically, the present invention relates to osteoblastogenesis and osteoclastogenesis and the regulation of these processes to control bone remodeling in adult life.

Description of the Related Art

Bone remodeling, a process responsible for the renewal of the adult human skeleton approximately every ten years, is carried out by teams of juxtaposed osteoclasts and osteoblasts - two specialized cell types that originate from hematopoietic and mesenchymal progenitors of the bone marrow, respectively (1,2). Continuous and orderly supply of these cells is essential for skeletal homeostasis, as increased or decreased production of osteoclasts or osteoblasts and/or changes in the rate of their apoptosis are largely responsible for the imbalance between bone resorption and formation that underlies several systemic or localized bone diseases such as osteoporosis, Paget's disease, metastatic disease and renal bone disease (3-9). Heretofore, little was known about the factors responsible for sustaining the supply of osteoblasts in postnatal life and how osteoblastogenesis and osteoclastogenesis are coordinated in order to assure a balance between formation and resorption during normal bone remodeling.

Bone morphogenetic proteins (BMPs), members of the TGFβ (transforming growth factor β) superfamily of proteins, are unique among growth factors that influence osteoblast differentiation because they can initiate this process from uncommitted progenitors *in vitro* as well as *in vivo* (10-12). In particular, BMP-2 and -4 are expressed during murine embryonal skeletogenesis (day 10-12) and act on cells isolated from murine limb buds to promote their differentiation into osteoblasts. In addition, BMP-2 and -4 are involved in fracture healing, as evidenced by their expression in primitive mesenchymal cells and chondrocytes at the site of callus formation; and the ability of

bone morphogenetic proteins to accelerate the fracture healing process when supplied exogenously (10,11). Besides BMPs -2 and -4, BMPs -5, -6 and -7 may also contribute to osteoblastic cell differentiation and bone formation (10). BMP-2/4-induced osteoblast commitment is mediated by the type I BMP receptor and involves the phosphorylation of specific transactivators (smad 1, 5 and 8), which then oligomerize with smad 4, and translocate into the nucleus (13). These events induce an osteoblast specific transcription factor [CBFA-1 (core binding factor-1), also known as Osf-2, PEBP2aA and AML3], which in turn activates osteoblast-specific genes (14,15).

Several proteins able to antagonize bone morphogenetic protein action have recently been discovered. Noggin, chordin and cerberus were initially found in the Spemann organizer region of the *Xenopus* embryo and were shown to be essential for neuronal or head development (16-21). Noggin and chordin inhibit the action of bone morphogenetic proteins by binding to them with high affinity and preventing their interaction with their receptors. Of the bone morphogenetic proteins tested, noggin displays specificity, in that binding is very tight to BMP-2 and BMP-4 (Kd = 2 x 10^{-11} M), weak to BMP-7, and undetectable to TGFβ or IGF-1 (insulin-like growth factor-1) (17).

Osteoclastogenesis and osteoblastogenesis proceed simultaneously in most circumstances (4,8), and the former may not occur without the latter (15,22) because osteoclast development requires support from stromal/osteoblastic cells (23). The mechanistic basis of this dependency has been recently explained by the discovery of a membrane bound cytokine-like molecule, receptor activator of NF-κB ligand (RANKL), which is

present in mesenchymal cells and binds to a specific receptor (RANK) on hematopoietic osteoclast progenitors (24-26). Such binding is essential, and, together with M-CSF, sufficient for osteoclastogenesis.

Thus, the prior art is deficient in understanding the balance between osteoclastogenesis and osteoblastogenesis in the adult skeleton. Furthermore, the prior art is deficient in compounds and methods that regulate bone homeostasis, and therefore, bone remodeling. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

Herein, the BMP-antagonist properties of noggin have been exploited to test the hypothesis that bone morphogenetic proteins are involved in the osteoblastogenesis that takes place in the bone marrow in postnatal life. It is shown herein that noggin does indeed inhibit both osteoblast and osteoclast formation in the postnatal murine bone marrow. Consistent with these observations, it was also demonstrated that the genes for BMP-2 and -4, the BMP-2/4 receptor, as well as noggin, are expressed in the adult murine bone marrow, a marrow-derived stromal/osteoblastic cell line found in murine adult whole bone.

One object of the present invention is to provide various methods of regulating bone remodeling in the adult skeleton. In an embodiment of the present invention, there is provided a method of suppressing excessive bone resorption in an individual, which comprises administering a therapeutic amount

of an antagonist of a bone morphogenetic protein to the individual, wherein the antagonist of a bone morphogenetic protein suppresses excessive bone resorption.

In another embodiment of the present invention, there is provided a method of increasing bone density in an individual, comprising: administering a therapeutic amount of an antibody directed towards an antagonist of a bone morphogenetic protein. The antibody interferes with the antagonist of a bone morphogenetic protein, thereby stimulating osteoblastogenesis and osteoclastogenesis and increasing bone density.

In yet another embodiment of the present invention, there is provided a method of regulating bone remodeling in an individual, comprising: administering an effective amount of either or both: one or more antagonists of one or more bone morphogenetic proteins; and/or at least one antibody directed towards the antagonist(s) or directed towards one or more different antagonists. The administration of the antagonist(s) results in a decrease of bone resorption, and administration of the antibody or antibodies results in an increase in bone density.

In still yet another embodiment of the present invention, there is provided a method of regulating the action of bone morphogenetic protein-2 (BMP-2), comprising the step of: contacting the BMP-2 with either or both: noggin; and/or at least one antibody directed towards the noggin. The administration of the noggin results in an inhibition of BMP-2, wherein administration of the antibody results in an activation of BMP-2.

Other and further aspects, features, and advantages of the present invention will be apparent from the following

description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the effect of noggin on BMP-2- or BMP-6-induced alkaline phosphatase in C2C12 cells. Cells (2×10^4 per cm^2) were cultured for 3 days in the presence of the indicated concentrations of BMP-2, -6 or noggin and alkaline phosphatase (AP) activity was measured. Each bar represents the mean (± SEM) of quadruplicate determinations. Data were analyzed by ANOVA after establishing homogeneity of variances. *$P<0.05$ versus cells cultured in 100 ng/ml BMP-2 alone.

Figure 2 shows the effect of noggin on osteoblastogenesis. Bone marrow cells were maintained in the absence or presence of the indicated concentrations of recombinant human noggin, without or with 100 ng/ml human

recombinant BMP-2. The total number of CFU-F colonies (**Figure 2A**) or the number of AP-positive CFU-F colonies (**Figure 2B**) were determined after 10 days of culture by staining for AP, while the number of CFU-OB colonies (**Figure 2C**) was determined after 28 days of culture by Von Kossa staining. Data shown are the mean number of colonies (SEM) per 10 cm^2 well (N=4 wells per group). In the presence of 100 ng/ml noggin, AP activity (Sigma kit #104. St. Louis, MO) decreased from 15.0 ± 4.0 to 0.5 ± 0.1 nmol p-Nitrophenol hydrolyzed/min/mg protein ($P < 0.01$ by Students t-test) in CFU-F colonies formed in 10 day cultures of murine bone marrow cells. **Figure 2D** shows UAMS-33 cell differentiation. Data shown are the mean (± SEM) AP activity after correction for cellular protein. Inset: one day after initiation of culture ('0'), cells were maintained in the absence (open circles) or presence (closed circles) of 100 ng/ml PEG-noggin for 4 days. In a subset of cultures, medium was replaced at 4 days and cultures continued for another 4 days without or with PEG-noggin; in addition, PEG-noggin was removed from medium of some cultures by replacing medium without PEG-noggin (closed triangle). AP activity was determined at 0, 4 and 8 days in parallel cultures. Data were analyzed by ANOVA. *$P < 0.05$ versus cells cultured without noggin or PEG-noggin; (cross) $P<0.05$ versus cells cultured without noggin, or cells cultured with noggin and BMP-2; (double cross) $P<0.05$ vs. cells maintained for 8 days with PEG-noggin.

Figure 3 shows the effect of PEG-noggin and BMP-2 on osteoclast formation. **Figure 3A** and **3B** show bone marrow cells from the femurs of 3-4 month old mice were maintained in the absence or the presence of (A) 10^{-8} M $1,25(OH)_2D_3$ or (B) 10^{-8}

M human PTH (1-34) without or with 200 ng/ml PEG-noggin or 300 ng/ml BMP-2. **Figure 3C** and **3D** show co-cultures of nonadherent bone marrow cells (1 x 10^6 cells per well) and either UAMS-33 cells (2 x 10^4 per well) (**C**) or murine calvaria cells (2 x 10^4 per well) (**D**) were stimulated with 10^{-8} M $1,25(OH)_2D_3$ or PTH, respectively. Osteoclastic cells were visualized by TRAP staining (N=4 per group). Data shown are the mean number (± SEM) of multinucleated TRAP-positive cells per well. Data were analyzed by ANOVA. *P <0.05 versus cultures stimulated with $1,25(OH)_2D_3$ or PTH alone.

Figure 4 shows that noggin does not effect hematopoietic cell differentiation toward the osteoclast phenotype. Non-adherent murine bone marrow cells were cultured for 6 days in the presence of the indicated reagents and osteoclast development was determined by TRAP staining. Data shown are the mean number (± SEM) of multinucleated TRAP-positive cells per well.

Figure 5 shows a decrease of RANKL mRNA abundance by PEG-noggin. UAMS-33 cells were cultured for 3 days in the absence (Cont) or presence of 10^{-8} M $1,25(OH)_2D_3$ or 200 ng/ml PEG-noggin alone or in combination, and Northern analysis was performed using total RNA. GAPDH, a housekeeping enzyme, was used as a control for loading and the ratio of the mRNA of interest and GAPDH is shown on top of the corresponding lanes.

Figure 6 shows expression of BMPs, noggin and BMP receptors in a murine pre-osteoblast cell line, cultured bone marrow cells, and bone homogenates. (**Figure 6A**) RNA was prepared from freshly isolated bone marrow cells (BMC), or from

2, 4, 7, 14 and 21 day cultures of these cells. (**Figure 6B**) Two week cultures of murine bone marrow cells were established and after fixation osteocalcin transcripts were detected in by *in situ* RT-PCR. As a negative control, parallel cultures were processed for BMP-4 detection without reverse transcriptase. Original magnification = 100X. (**Figure 6C**) RNA was prepared from two week cultures of murine bone marrow cells, and from 3 day cultures of UAMS-33 cells and assayed by RNase protection for BMP-4 and BMPR-IA transcripts. The expected bands for BMP-4, BMPR-IA and β-actin are 290, 216 and 174 bp, respectively, and are shown by arrows. Intact RNA probes used in this assay exhibit a higher molecular weight than the protected bands, and are absent after RNase digestion. (**Figure 6D**) Two week cultures of murine bone marrow cells, and 3 day cultures of UAMS-33 cells, were immunohistostained with an antibody against BMP-2 and -4 (+Ab). As a negative control, cells were processed without primary antibody (-Ab). Original magnification = 100X. (**Figure 6E**) (Left and Middle panel) RNA was prepared from femurs of neonatal, or brain and femurs of 3 month old Swiss Webster mice and analyzed for BMP-4, noggin, and GAPDH transcripts by RT-PCR. (Right panel) Northern analysis of polyA+ RNA from 6 day cultures of UAMS-33 cells. (**Figure 6F**) (Left panel) Western blot analysis of noggin in lysates from UAMS-33 cells cultured for 0, 3 or 6 days, from murine bone marrow cells cultured for two weeks, and from intact femurs of 3 month old Swiss Webster mice. As a positive control, 10 ng of recombinant human noggin (rNoggin) was used. (Right panel) Western blot analysis of BMP-2/4 in homogenates of adult murine femoral bone. As a positive control, 50 ng of recombinant human BMP-2 (rBMP-2) was used.

DETAILED DESCRIPTION OF THE INVENTION

Bone morphogenetic proteins (BMPs) have been heretofore implicated in the induction of osteoblast differentiation from uncommitted progenitors during embryonic skeletogenesis and fracture healing. The present invention demonstrates that bone morphogenetic proteins are also involved in the osteoblastogenesis that takes place in the bone marrow in postnatal life. Noggin, a recently discovered protein that binds bone morphogenetic proteins -2 and -4 and blocks their action, was used to inhibit osteoblastogenesis. Addition of human recombinant noggin to bone marrow cell cultures from normal adult mice inhibited not only osteoblast, but also osteoclast formation; these effects were reversed by exogenous BMP-2. Consistent with these findings, BMP-2 and -4 and BMP-2/4 receptor transcripts and proteins were detected in these primary cultures, in a bone marrow derived stromal/osteoblastic cell line, as well as in murine adult whole bone; noggin expression was also documented in all these preparations. These findings provide evidence that BMPs -2 and -4 are expressed in the bone marrow in postnatal life and serve to maintain the continuous supply of osteoblasts and osteoclasts. Additionally, BMP-2/4-induced commitment to the osteoblastic lineage is a prerequisite for osteoclast development. Hence, BMPs - perhaps in balance with noggin and possibly other antagonists - may provide the tonic baseline control of the rate of bone remodeling upon which other inputs (*e.g.*, hormonal, biomechanical, etc.) operate.

Thus, the present invention is directed towards methods of regulating postembryonic bone remodeling, both in stimulating and inhibiting osteogenesis as necessary.

It is an object of the present invention to provide a method of suppressing excessive bone resorption in an individual, comprising: administering a therapeutic amount of an antagonist of a bone morphogenetic protein to the individual, wherein the antagonist of a bone morphogenetic protein suppresses excessive bone resorption. Representative diseases for which this method may be used include postmenopausal osteoporosis, rheumatoid arthritis, hyperparathyroidism, primary hyperparathyroidism, secondary hyperparathyroidism, multiple myeloma, periodontal disease, Gorham-Stout disease and McCune-Albright syndrome.

It is another object of the present invention to provide a method of increasing bone density in an individual, comprising: administering a therapeutic amount of an antibody directed towards an antagonist of a bone morphogenetic protein. The antibody interferes with the antagonist, which thereby stimulates osteoblastogenesis and osteoclastogenesis and increases bone density. Representative diseases the individual may suffer from include senescence-associated osteoporosis and adynamic bone disease.

The present invention further provides for a method of regulating bone remodeling in an individual, comprising the steps of: administering an effective amount of either or both: one or more antagonists of one or more bone morphogenetic proteins; and/or at least one antibody directed towards the antagonist(s) or directed towards one or more different antagonists.

Administration of the antagonist(s) results in a decrease of bone resorption, wherein administration of the antibody or antibodies results in an increase in bone density. Diseases for which this method may be will be readily recognized by those having ordinary skill in this art.

In the above-mentioned objectives, representative antagonists are noggin, chordin and cerberus, and preferably, representative bone morphogenetic protein are BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-12 or BMP-13. It is additionally an object of the present invention to provide a method of regulating the action of bone morphogenetic protein-2 (BMP-2), comprising the step of: contacting the BMP-2 with either or both: noggin; and at least one antibody directed towards noggin. Administration of noggin results in an inhibition of BMP-2, while administration of the antibody results in an activation of BMP-2.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, "Molecular Cloning: A Laboratory Manual (2nd Ed.)", (1989); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning"

(1984). Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein, the term "antagonist of a bone morphogenetic protein" refers to a protein that interferes with the ability of bone morphogenetic proteins to interact with their receptors on the surface of target cells.

As used herein, the term "bone density" refers to the amount of mineralized tissue per unit volume of anatomical bone.

As used herein, the term "bone morphogenetic protein" refers to a family of acidic proteins which are able to stimulate cells to differentiate into bone cells.

As used herein, the term "excessive bone resorption" refers to abnormal loss of bone mass leading to deterioration of it's structural integrity.

As used herein, the terms "osteoblastogenesis" and "osteoclastogenesis" refers to the generation and/or development of osteoblast and osteoclast cells. Osteoblasts originate from mesenchymal stem cells, while osteoclasts originate from hematopoietic stem cells.

It is specifically contemplated that pharmaceutical compositions may be prepared using noggin or human recombinant noggin (rNoggin). In such a case, the pharmaceutical composition comprises noggin or rNoggin and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of noggin or rNoggin. When used *in vivo* for therapy, noggin or rNoggin is administered to the patient or an

animal in therapeutically effective amounts, *i.e.*, amounts that inhibit bone morphogenetic proteins and thereby inhibit excessive bone resorption. It will normally be administered parenterally, preferably intravenously, but other routes of administration will be used as appropriate. The dose and dosage regimen will depend upon the extent of bone remodeling desired, the patient, the patient's history and other factors. The amount of noggin or rNoggin administered will typically be in the range of about 10^{-9} to about 10^{-6} g/kg of patient weight. The schedule will be continued to optimize effectiveness while balanced against negative effects of treatment. *See* Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Penn.; and *Goodman and Gilman's: The Pharmacological Basis of Therapeutics* 8th Ed (1990) Pergamon Press. For parenteral administration, the noggin or rNoggin will most typically be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, *e.g.*, buffers and preservatives.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Materials

Human recombinant BMP-2, -4, -6, -12 (GDF-7), and -13 (GDF-6) and an anti-human BMP-2/4 antibody, which recognizes BMP-2 and -4, were provided by V. Rosen (Genetic Institute, Cambridge, MA). $1,25(OH)_2D_3$ and murine soluble RANKL were provided by Hoffmann-LaRoche (Nutley, NJ) and Dr. B. Boyle (Amgen Inc., Thousand Oaks, CA), respectively. Human PTH (parathyroid hormone)(1-34) and human recombinant M-CSF (macrophage-colony stimulating factor) were purchased from Peninsula Laboratories (Belmont, CA) and R & D Systems (Minneapolis, MN), respectively. cDNA probes for murine BMP-4 and BMP receptor type IA (BMPR-IA) were provided by Dr. K Miyazono (Cancer Institute, Tokyo, Japan). A murine RANKL cDNA probe was provided by Immunex Corp. (Seattle, Washington).

EXAMPLE 2

Osteoblast progenitor assays

C2C12 cells (2×10^4 per cm^2), a murine myoblastic line that can differentiate into osteoblastic cells upon stimulation with exogenous BMP-2 or to myotubes under low serum concentration (27), were cultured with or without 100 ng/ml BMP-2 or BMP-6 and/or 10-600 ng/ml noggin for 3 days. Alkaline phosphatase (AP) activity was measured by Sigma kit #104. Bone marrow cells were obtained from femurs of 3 month old male Swiss Webster mice and cultured at 1×10^6 cells (for CFU-F determination) or 2×10^6 cells (for CFU-OB) per 10 cm^2 well in αMEM (α-minimal essential medium, Gibco-BRL, Gaithersburg, MD) supplemented

with 15% preselected FCS (Hyclone, Logan, UT), 200 mM ascorbic acid, and 10 mM β-glycerophosphate. Cultures were maintained in the absence or presence of different concentrations of recombinant human noggin, without or with 100 ng/ml human recombinant BMP-2. The total number of CFU-F colonies and the number of AP-positive CFU-F colonies were determined after 10 days of culture by staining for AP, and the number of CFU-OB colonies was determined after 28 days of culture by Von Kossa staining (8). UAMS-33, a cell line with stromal/osteoblastic properties, was obtained by limiting dilution subcloning from foci of transformed cells that developed during long term culture of murine bone marrow cells (28). For the osteoblast differentiation experiments, UAMS-33 cells were plated at 2 x 10^4 per cm^2 well and maintained for up to 8 days in αMEM containing 10% FCS in the presence of the indicated concentrations of recombinant human noggin or a PEG-ylated noggin (PEG-noggin). Osteoblast differentiation was assessed by measuring AP activity.

EXAMPLE 3

Osteoclast development assays

Bone marrow cells were obtained from the femurs of 3-4 month old mice and cultured for 8 days for the determination of osteoclast formation (3). Cultures were maintained in the absence or the presence of 10^{-8} M $1,25(OH)_2D_3$ or 10^{-8} M human PTH (1-34) without or with 200 ng/ml PEG-noggin or 300 ng/ml BMP-2. In the experiments shown in Figure 2C and 2D, osteoclast formation was assessed in 8 day long co-cultures of non-adherent bone marrow cells (1 x 10^6 cells per well) and either UAMS-33 cells (2 x 10^4 per well) or murine calvaria cells (2 x 10^4 per well).

Direct effects of noggin on hematopoietic cell differentiation toward the osteoclastic phenotype were examined using non-adherent murine bone marrow cells (10^5 per well). Non-adherent bone marrow cells obtained by preculturing bone marrow cells for 2 days (in order to remove stromal/osteoblasts) were cultured for 6 days with 10 ng/ml human M-CSF, 100 ng/ml soluble RANKL, and 10 or 100 ng/ml PEG-noggin. In all these experiments, osteoclastic cells were visualized by staining for tartrate resistant acid phosphatase (TRAP) using Sigma kit #180.

EXAMPLE 4

RT-PCR analysis

RNA was prepared from freshly isolated bone marrow cells, or from 2, 4, 7, 14 and 21 day cultures (29) and analyzed for the expression of BMP-2, BMP-4, osteocalcin, BMPR (BMP receptor)-IA, and BMPR-IB transcripts by RT-PCR. RT-PCR was performed using primers, as detailed previously for GAPDH (glyceraldehyde-3-phosphate dehydrogenase)(29). Primer sets were as follows:

For murine BMP-2:

5'- CTAGTGTTGCTGCTTCCCCA (forward) (SEQ ID No. 1),

5'- GAGTTCAGGTGGTCAGCAAG (reverse) (SEQ ID No. 2);

For murine BMP-4:

5'- GCGCCGTCATTCCGGATTAC (forward) (SEQ ID No. 3),

5'- CATTGTGATGGACTAGTCTG (reverse) (SEQ ID No. 4);

For murine BMPR-IA:

5'- GGCAGAATCTAGATAGTATGCTCC (forward) (SEQ ID No. 5),

5'- GAAGTTAACGTGGTTTCTCCCTG (reverse) (SEQ ID No. 6);

For murine BMPR-IB:

5'- CACCAAGAAGGAGGATGGAGAGA (forward) (SEQ ID No. 7),

5'- CTACAGACAGTCACAGATAAGC (reverse) (SEQ ID No. 8);

For murine osteocalcin:

5'-TCTGACAAAGCCTTCATGTCC (forward) (SEQ ID No. 9),

5'-AAATAGTGATACCGTAGATGCG (reverse) (SEQ ID No. 10);

For murine noggin:

5'- TGGACCTCATCGAACATCCAGAC (forward) (SEQ ID No. 11),

5'- ACTTGGATGGCTTACACACCATGC (reverse) (SEQ ID No. 12).

Using these primers, the expected sizes of the PCR products are as depicted in Figure 6A.

EXAMPLE 5

In situ RT-PCR

Bone marrow cultures were established and maintained for two weeks. After fixation with 4% paraformaldehyde, BMP-4 and osteocalcin transcripts were detected using the BMP-4 and osteocalcin primers described in Example 4 above (29). As a negative control, parallel cultures were processed for BMP-4 detection without reverse transcriptase.

EXAMPLE 6

RNase protection assay

RNA was prepared from two week cultures of murine bone marrow cells, and from 3 day cultures of UAMS-33 cells and assayed by RNase protection for BMP-4 and BMPR-IA transcripts. For the preparation of the cDNA probes, plasmids containing the coding regions of murine BMP-4 and BMPR-IA were subcloned in

Bluescript KS(+) plasmids and linearized. The respective riboprobes were synthesized in the presence of 50-100 mCi of [^{32}P]-UTP (3000 Ci/mmol, Amersham Corp., Arlington Heights, IL), and T7 or SP6 RNA polymerase, as appropriate (Promega, Madison, WI). Total RNA (30 µg) was extracted from 3 day cultures of UAMS-33 cells and 2 week old cultures of murine bone marrow cells. RNA and ^{32}P-labeled riboprobes in hybridization buffer (80% formaldehyde, 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA) were annealed at 45°C overnight after heating at 85°C for 5 min. Subsequently, annealed RNAs were treated with RNase A (40 µg/ml) at 30°C for 60 min, and the enzyme was inactivated by proteinase K (100 µg) and 10% SDS. Finally, the samples were loaded on 4.5% polyacrylamide gels with 7 M urea after extraction by phenol and ethanol.

EXAMPLE 7

Immunostaining

Two week cultures of murine bone marrow cells or 3 day cultures of UAMS-33 cells were immunohistostained with an antibody against BMP-2 and -4. Cells were fixed with 10% formalin for 10 minutes, treated with 0.1% H_2O_2 for 30 minutes to remove endogenous peroxidase activity, and blocked with 5% normal goat serum for 1 hour. They were then incubated with mouse anti-human BMP-2/4 antibody for 1 hour and subsequently for 30 min with a biotinylated second antibody (Vector, Burlingame, CA), followed by incubation with peroxidase-conjugated streptavidin, and 3,3'-diaminobenzidine tetrachloride (Santa Cruz Biotechnology Inc., Santa Cruz, CA). As a negative control, cells were processed without primary antibody.

EXAMPLE 8

Northern blot analysis

Total RNA (30 mg) or polyA+ RNA (8 mg) were prepared from 3 or 6 day cultures of UAMS-33 cells, and were electrophoresed on 1% agarose gels. Northern blotting for RANKL, M-CSF and noggin expression were performed (29).

EXAMPLE 9

Western blot analysis

Cells were lysed in 10 mM phosphate buffer (pH 7.4), 10% glycerol, 1% NP-40, 0.1% SDS, 4 mM EDTA, 0.15 M NaCl, 0.01 M NaF, 0.1% sodium orthovanadate, 1 mM PMSF, 5 mg/ml trypsin inhibitor, and 5 mg/ml protease inhibitors, and centrifuged at 14,000 x g for 10 min. For preparation of bone homogenates, femurs were frozen in liquid N_2 and pulvarized. The powder was homogenized in the above buffer using a Polytron homogenizer, and then centrifuged. Supernatant proteins (50 µg) from the cell lysates or the bone homogenates were subjected to 12% SDS-PAGE (polyacrylamide gel electrophoresis) and electroblotted onto a PVDF membrane (Millipore, Bedford, MA). Membranes were incubated for 2 h at room temperature in 5% dry milk in 20 mM Tris (pH 7.2), 0.15 M NaCl containing 0.05% Tween 20, and subsequently with anti-BMP 2/4 antibody, or the anti-noggin antibody, and then appropriate second antibody [HRPO (horseradish peroxidase)-goat anti-mouse IgG antibody for BMP-2/4 or HRPO-goat anti-rat IgG antibody for noggin]. The bound antibody on the membrane was detected by enzyme reaction

using an enhanced chemiluminescence kit (Dupont NEN, Boston, MA) (29).

EXAMPLE 10

Inhibition of osteoblastogenesis by noggin

The ability of human recombinant noggin to antagonize BMP-mediated commitment to the osteoblast lineage, as well as the specificity of this effect, was established using C2C12 cells (27). Human recombinant noggin (100-600 ng/ml) inhibited BMP-2-induced alkaline phosphatase (AP) activity, a phenotypic marker of osteoblastic cells, in a dose dependent manner (Figure 1). At the highest concentration of noggin, the effect of BMP was completely blocked. Like BMP-2, BMP-6 stimulated alkaline phosphatase activity in C2C12 cells; however, the effect of BMP-6 was not affected by noggin, indicating that noggin is not an antagonist of BMP-6. BMP-12 or -13 alone did not induce alkaline phosphatase activity in C2C12 cells, nor did they interfere with the stimulatory effect of BMP-2 or -6, thereby confirming previous reports (30).

Noggin had no effect on the number of colony forming units-fibroblast (CFU-F) that were formed during a 10-day long primary culture of murine bone marrow cells obtained from 3-month-old mice (Figure 2A). Noggin, however, led to a dose dependent decrease in the number of alkaline phosphatase positive CFU-F colonies (Figure 2B). Noggin also inhibited bone nodule formation by subsets of CFU-F colonies, designated CFU-osteoblast (CFU-OB), in 28 day-long primary cultures of bone marrow cells (Figure 2C). Both effects were detectable with as little as 6-10 ng/ml of noggin. Practically complete suppression of

alkaline phosphatase expression in CFU-F or bone nodule formation by noggin could be seen with 150-600 ng/ml. The inhibitory effect of noggin on alkaline phosphatase expression in CFU-F colonies could be reversed by addition of 100 ng/ml BMP-2, except at the highest concentration of noggin (600 ng/ml), confirming competitive antagonism between noggin and BMPs (Figure 2B). Similarly, noggin dose-dependently reduced alkaline phosphatase activity in UAMS-33 cultures maintained under basal conditions (Figure 2D); or in the presence of exogenous BMP-2. Noggin also prevented calcium deposition by UAMS-33 cells cultured in the presence of ascorbic acid and β-glycerophosphate for 5 days. In all these experiments, PEG-noggin (prepared by covalently linking polyethyleneglycol to the lysine residues of noggin) was more potent than noggin, most likely due to increased stability of the protein. Withdrawal of PEG-noggin after 4 days restored alkaline phosphatase levels in UAMS-33 cells, demonstrating that the effects of the protein seen herein were not due to cytotoxic actions (Figure 2D). Consistent with this, noggin had no effect on cell viability as measured by trypan blue exclusion.

EXAMPLE 11

Inhibition of osteoclastogenesis by noggin

Osteoclast formation induced by either $1,25(OH)_2D_3$ or PTH was also inhibited by the addition of PEG-noggin in bone marrow cell cultures (Figures 3A and 3B), or co-cultures of non-adherent bone marrow cells, a source of osteoclast progenitors, and either UAMS-33 cells (Figure 3C) or neonatal murine calvaria osteoblastic cells (Figure 3D); UAMS-33 cells can support osteoclast

differentiation induced by $1,25(OH)_2D_3$, but unlike calvaria cells, they do not support PTH-induced osteoclast development. The effect of noggin was dose dependent and identical in experiments using cells from 3 or 6 month old mice. In contrast, exogenous human recombinant BMP-2 increased osteoclast formation as much as 4-6 fold over baseline in PTH-stimulated primary bone marrow cell cultures (Figure 3B), and in co-cultures of hematopoietic progenitors and calvaria cells (Figure 3D). An increase, albeit smaller, was also observed in $1,25(OH)_2D_3$-stimulated cultures (Figures 3A and 3C). However, in different experiments wherein co-cultures of UAMS-33 cells and non-adherent hematopoietic precursors were pretreated with BMP-2 prior to exposure to $1,25(OH)_2D_3$, BMP-2 stimulated osteoclast formation by 3-4 fold.

To investigate the possibility that noggin could have inhibited osteoclast formation in part as a result of direct actions on hematopoietic cells, preparations of non-adherent murine bone marrow cells (devoid of stromal/osteoblastic cells) were cultured for 6 days in the presence of human recombinant M-CSF and soluble murine RANK ligand (sRANKL)(Figure 4). As shown previously by Lacey et al (24), sRANKL-along with M-CSF was sufficient for the induction of osteoclast formation in the absence of stromal/osteoblastic support cells or stimuli like $1,25(OH)_2D_3$ or PTH. In this system of stromal/osteoblastic cell-independent osteoclastogenesis, noggin had no effect on osteoclast development. Consistent with the conclusion that noggin does not exert its anti-osteoclastogenic effects through direct actions on hematopoietic osteoclast precursors, PEG-noggin had no effect on

M-CSF stimulated proliferation of non-adherent hematopoietic progenitors isolated from murine bone marrow.

Consistent with the notion that the effect of noggin on osteoclastogenesis was due to attenuation of stromal/osteoblastic cell differentiation toward a state capable of supporting osteoclast development, PEG-noggin at 200 ng/ml decreased the expression of the mRNA for RANK ligand by 40% in $1,25(OH)_2D_3$-treated UAMS-33 cells (Figure 5). However, at the same concentration, PEG-noggin had no effect on M-CSF mRNA. Besides affecting the differentiated phenotype, the inhibitory effects of PEG-noggin on osteoclast development could be caused by a reduction in the total number of stromal/osteoblastic cells. This possibility was excluded by showing that PEG-noggin did not affect UAMS-33 or calvaria cell proliferation under the conditions used in the co-cultures of Figure 3.

EXAMPLE 12

Expression of BMPs-2/4, their receptors and noggin in bone marrow and bone

The results presented herein strongly suggested that the genes encoding bone morphogenetic proteins and their receptors are expressed in bone marrow cultures from normal adult mice and that their products are required for osteoblastogenesis as well as osteoclastogenesis. Therefore, BMP-2, -4, and -7 transcripts and proteins were examined in bone marrow cells and homogenates of bone from femurs of three month old mice (Figure 6). BMP-4 transcripts could be detected by RT-PCR in adherent bone marrow cells maintained in culture as early as 4 days and BMP-2 transcripts in cells maintained for 2

weeks; but not in freshly isolated bone marrow cells (Figure 6A). In contrast to BMP-2 and -4, BMP-7 transcripts were not detected in either freshly isolated or cultured bone marrow cells. BMP receptor type IA was detected by RT-PCR in freshly isolated cells and throughout the culture; whereas the type IB receptor was first visualized at 4 days. Osteocalcin mRNA was first detected at 2 weeks of culture. It was previously demonstrated that IL-6, gp130, and osteopontin transcripts are absent from freshly isolated bone marrow cells but appear during culture (29,31). Hence, the expression of bone morphogenetic proteins and BMP receptor genes upon culture of bone marrow cells is most likely the result of an expansion of the osteogenic cell population. Using *in situ* RT-PCR analysis, the BMP-4 and osteocalcin transcripts were localized in a subset of cells present within the CFU-F colonies (Figure 6B). Independent confirmation of the RT-PCR results for BMP-4 and BMPR-IA expression in bone marrow cultures was obtained using RNase protection assay of RNA isolated from 2 week long cultures; identical transcripts were also seen in the UAMS-33 cells (Figure 6C). In agreement with the mRNA studies, the BMP-2/4 proteins were detected in bone marrow cells cultured for 2 weeks as well as in UAMS-33 cells by immunostaining with an antibody that recognizes both BMP-2 and -4 (Figure 6D). Moreover, BMP-4 transcripts and BMP-2/4 proteins as well as noggin transcripts and proteins could be demonstrated by RT-PCR or Northern Blot analysis (Figure 6E) and Western Blot analysis (Figure 6F) in homogenates of whole femurs from 3 month old mice and also in UAMS-33 cells. In UAMS-33 cells, noggin expression was highest in post-confluent cultures (6

days), at which time the activity of the osteoblast phenotypic marker, alkaline phosphatase, was maximal.

Nature recapitulates evolutionarily successful mechanisms to accomplish similar tasks at different stages of life

The results of this study reinforce this truism by demonstrating that BMPs-2/4, the same proteins that have been implicated in the induction of osteoblast formation in the embryo and during fracture repair, are required for osteoblastogenesis in the murine bone marrow in postnatal life. In addition, the data presented herein reveal for the first time that induction of mesenchymal cell differentiation toward the osteoblast phenotype by BMPs-2/4 is a prerequisite for osteoclastogenesis. Elucidation of the requirement of bone morphogenetic proteins for osteoclastogenesis offers an entirely new perspective of how bone homeostasis is maintained during physiologic remodeling and explains the *in vivo* observations that osteoclastogenesis (4,8,15) and loss of bone (22) cannot occur without osteoblastogenesis.

The evidence that bone morphogenetic proteins are required for osteoblast and osteoclast development implies that in addition to their previously known roles in skeletal development and repair, these proteins may also be involved in bone remodeling. All these processes require osteoblasts and osteoclasts, but it appears that the production of these cells is governed by the same proteins throughout life; consequently, biomechanical and local signals must determine how they are deployed for different purposes. The evidence that the genes for BMPs-2/4 and noggin are expressed in the adult murine bone marrow raises the possibility that the balance between bone

morphogenetic proteins and noggin provides a tonic baseline control of osteoblastogenesis and osteoclastogenesis upon which other inputs (*e.g.*, biomechanical, hormonal, etc.) operate, either by influencing the balance between bone morphogenetic proteins and noggin or by providing independent signals that alter the pro-differentiating effects of bone morphogenetic proteins. Noggin, however, may be just one of several bone morphogenetic protein antagonists with a role in the regulation of osteoblastogenesis and osteoclastogenesis, since other proteins such as chordin, have similar BMP antagonist properties (16-21).

In situ RT-PCR analysis of the bone marrow cultures suggests that the cells expressing BMPs-2/4 and noggin in bone marrow and intact bone are of the mesenchymal/osteoblastic lineage. Consistent with this, BMP-4, BMPR-IA, as well as noggin transcripts and proteins were detected in UAMS-33 cells, a bone marrow derived stromal/osteoblastic line (28). The level of expression of these genes in UAMS-33 cultures, a homogeneous preparation, was considerably higher as compared to the heterogeneous primary bone marrow cell cultures. This observation adds support to the contention that most of the cells expressing these genes in the bone marrow are of the mesenchymal/osteoblastic lineage. The observation that expression of noggin increased in the UAMS-33 cells during culture suggests that noggin expression by osteoblastic cells may increase as they advance to more differentiated stages. If noggin is produced by terminally differentiated osteoblasts, lining cells, or osteocytes, it could serve to restrict inappropriate osteoblastogenesis and thereby target remodeling to appropriate sites. It was recently shown that the absence of regulated BMP

activity in mice lacking noggin leads to failure of joint development (32), demonstrating that the balance between bone morphogenetic proteins and noggin has important biological implications; and further that bone morphogenetic proteins, as well as TGFβ, induce the expression of noggin in osteoblastic cells (33). Moreover, in line with the view that noggin serves to counteract the effects of BMPs *in vivo*, PEG-noggin administration to mice inhibits bone formation in subcutaneous implants of BMP-2-impregnated matrigel, a solubilized basement membrane preparation (34).

In agreement with the evidence that osteoblastogenesis is a prerequisite for osteoclastogenesis, highlighted herein by the evidence that the effects of noggin on osteoclastogenesis are due to antagonism of BMP actions on mesenchymal cells as opposed to direct actions on hematopoietic progenitors, it was previously determined that the promoters of both the murine and human RANK ligand gene contain two functional CBFA-1 binding sites; and that mutation of these sites abrogates the transcriptional activity of the RANKL promoter (35). Therefore, the BMP→CBFA-1→RANKL gene expression cascade in cells of the stromal/osteoblastic lineage likely constitutes the molecular basis of the linkage between osteoblastogenesis and osteoclastogenesis; with the last component probably requiring additional stimuli such as $1,25(OH)_2D_3$, PTH or gp130 activating cytokines.

In conclusion, the evidence presented in this study suggests that the balance between BMPs and their antagonists is a critical determinant of osteoblastogenesis as well as osteoclastogenesis, and therefore, the rate of bone remodeling.

Accordingly, it is possible that novel therapies for bone diseases may be developed based on the manipulation of the balance between these proteins.

EXAMPLE 13

Autocrine Regulation of Osteoblast Differentiation by Noggin

BMPs-2/4 and their antagonist, noggin, have been implicated in the control of adult bone remodeling based on evidence that they are both produced in the bone marrow, and when the latter is provided exogenously, it blocks osteoblastogenesis as well as osteoclastogenesis. The significance of endogenous noggin production by cells of the bone marrow and differentiated osteoblastic cells is elucidated by the following experiments. In murine bone marrow cell (BMC) cultures from 3 month old mice, the number of alkaline phosphatase (AP)-positive colony forming unit-fibroblast (CFU-F) or CFU-F exhibiting mineralization (CRU-OB) were significantly higher (35 and 100%, respectively) when maintained in the presence of a noggin-neutralizing antibody. Similarly, the noggin-neutralizing antibody caused a 25% increase in AP activity during 8 day long culture of primary osteoblastic cells (POB) from neonatal calvaria maintained in the presence of 300 ng/ml of human recombinant BMP-2. However, the antibody had no effect on AP activity in cultures that were not exposed to exogenous BMP-2, indicating that endogenously produced noggin was counteracting the effect of BMP-2. Independent confirmation of endogenous noggin production by osteoblastic cells was obtained with POB cells prepared from heterozygous fetuses of mice carrying a null-mutation in the noggin gene, in which the coding sequence was

replaced with the LacZ gene. β-galactosidase (β-gal) activity was increased in the presence of 300 ng/ml of human BMP-2 by 4-fold after 3 days of culture; however, it was not affected in cultures maintained in the presence of 5 ng/ml of transforming growth factor-β1. Consistent with these results, the number of β-gal positive cells increased time-dependently from 8.3% to 83.6% during 3 days of culture in the presence of BMP-2. By comparison, the number of β-gal positive cells was 3.6, 8.3 and 18.6% at 0, 3 and 6 days in POB maintained without exogenous BMP. These observations provide still further evidence that BMPs-2/4, in balance with noggin, are produced by cells of the osteoblastic lineage and act in an autocrine fashion to regulate osteoblast differentiation; and that a positive feedback loop of increased noggin expression by BMPs provides a counterregulatory mechanism to preserve this balance.

EXAMPLE 14

Demonstration of Noggin Expression by Bone Marrow Macrophages: A Paracrine Control of Osteoblast Differentiation

Noggin is expressed by chondrocytes prior to endochondral ossification, and a targeted disruption of the noggin gene causes defects in somite patterning and joint formation during embryogenesis. To probe further into the biologic role of noggin in the adult bone marrow, bone marrow cells (BMC) were obtained from heterozygous adult mice with a null mutation in the noggin gene, in which the coding sequence was replaced with the LacZ gene. Bone marrow cell cultures were maintained in α-MEM with 15% FBS in the presence of ascorbic acid and β-glycerophosphate for 1, 2 or 3 weeks. Noggin expressing cells,

identified by the expression of β-gal, were stained for alkaline phosphatase (AP), a phenotypic marker of stromal/osteoblastic cells, or for non-specific esterase (NSE), a marker of monocyte/macrophages.

Strikingly, throughout the culture period, the majority of noggin expressing cells also stained positive for NSE. On the other hand, there were very few, if any, noggin expressing AP(+)-stromal/osteoblastic cells up to 2 weeks of culture. However, by 3 weeks, such cells represented ~50% of the population. The evidence for noggin expression by bone marrow monocyte/macrophage-like cells was confirmed in enriched preparations of this cell type obtained by culturing non-adherent hematopoietic cells with 20 ng/ml of human M-CSF. Both the noggin transcript and protein could be easily identified by RT-PCR and Western blotting respectively, in these enriched preparations of marrow monocytes/macrophages as well as in a myeloid leukemia cell line (P388D1). Further, and in support of the evidence that noggin is indeed expressed by differentiated osteoblastic cells, it was determined that ~3.6% of cells isolated from 17 to 18 day old fetal calvaria from heterozygous noggin knockout/LacZ mice were expressing the transgene. It is concluded that noggin may regulate osteoblast differentiation in the marrow (by antagonizing BMP action) not only in an autocrine fashion, but also in a paracrine fashion, through its production by marrow macrophages.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method of suppressing excessive bone resorption in an individual in need of such treatment, comprising the step of:

administering a therapeutic amount of an antagonist of a bone morphogenetic protein to said individual, wherein said antagonist of a bone morphogenetic protein suppresses excessive bone resorption.

2. The method of claim 1, wherein said antagonist is selected from the group consisting of noggin, chordin and cerberus.

3. The method of claim 1, wherein said individual suffers from a metabolic bone disease selected from the group consisting of postmenopausal osteoporosis, reheumatoid arthritis, hyperparathyroidism, primary hyperparathyroidism, secondary hyperparathyroidism, multiple myeloma, periodontal disease, Gorham-Stout disease and McCune-Albright syndrome.

4. The method of claim 1, wherein said bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-12 and BMP-13.

5. A method of increasing bone density in an individual in need of such treatment, comprising the step of:

administering a therapeutic amount of an antibody directed towards an antagonist of a bone morphogenetic protein, wherein said antibody interferes with said antagonist, wherein said interference with said antagonist thereby stimulates osteoblastogenesis and osteoclastogenesis, wherein said stimulation increases bone density.

6. The method of claim 5, wherein said antagonist is selected from the group consisting of noggin, chordin and cerberus.

7. The method of claim 5, wherein said individual suffers from a disease selected from the group consisting of senescence-associated osteoporosis and adynamic bone disease.

8. The method of claim 5, wherein said bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-12 and BMP-13.

9. A method of regulating bone remodeling in an individual in need of such treatment, comprising the steps of:

administering an effective amount of either or both:

at least one antagonist of at least one bone morphogenetic protein; and

at least one antibody directed towards said antagonist(s) or directed towards one or more different antagonists, wherein administration of said antagonist(s) results in a decrease of bone resorption, wherein administration of said antibody or antibodies results in an increase in bone density.

10. The method of claim 9, wherein said antagonist is selected from the group consisting of noggin, chordin and cerberus.

11. The method of claim 9, wherein said bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-12 and BMP-13.

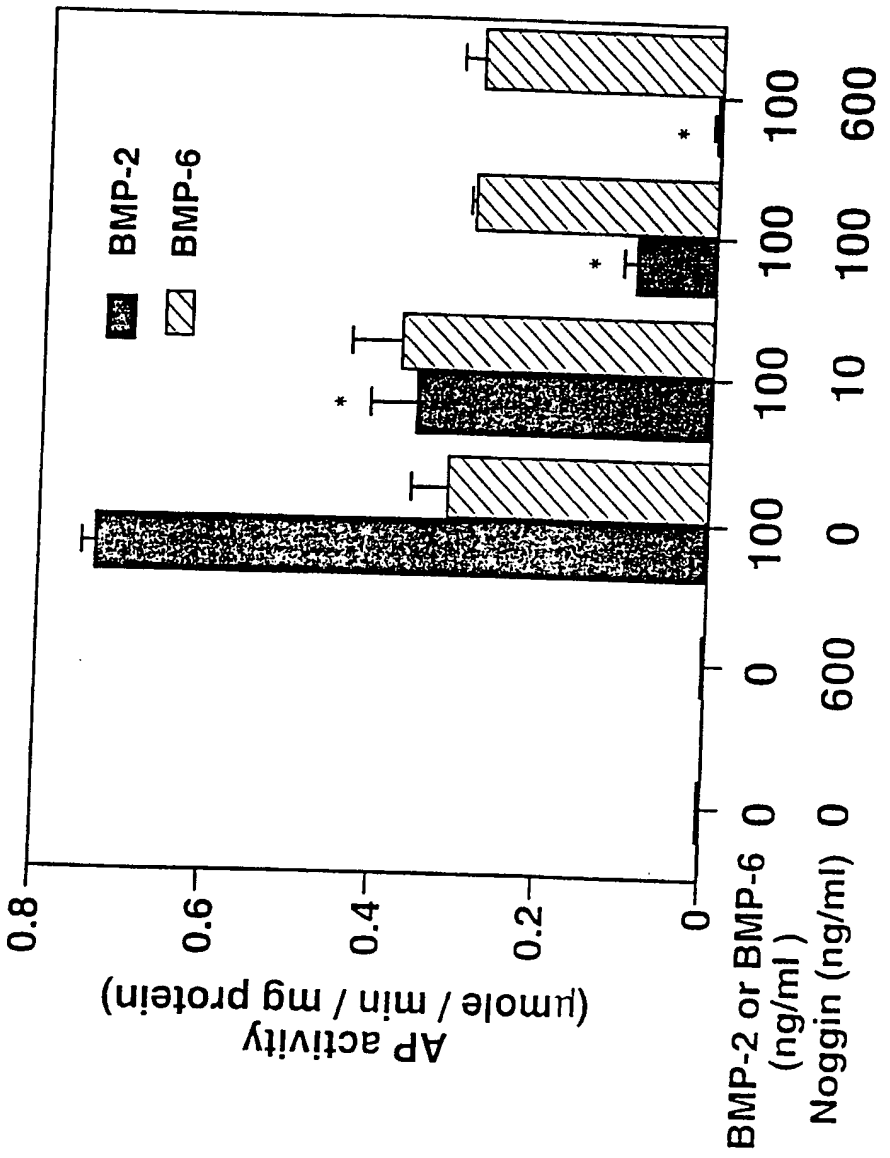
12. A method of regulating the action of bone morphogenetic protein-2 (BMP-2), comprising the steps of:

contacting said BMP-2 with either or both:

noggin; and

at least one antibody directed towards said noggin, wherein administration of said noggin results in an inhibition of BMP-2, wherein administration of said antibody results in an activation of BMP-2.

Figure 1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0.4
0.2
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BMP-6
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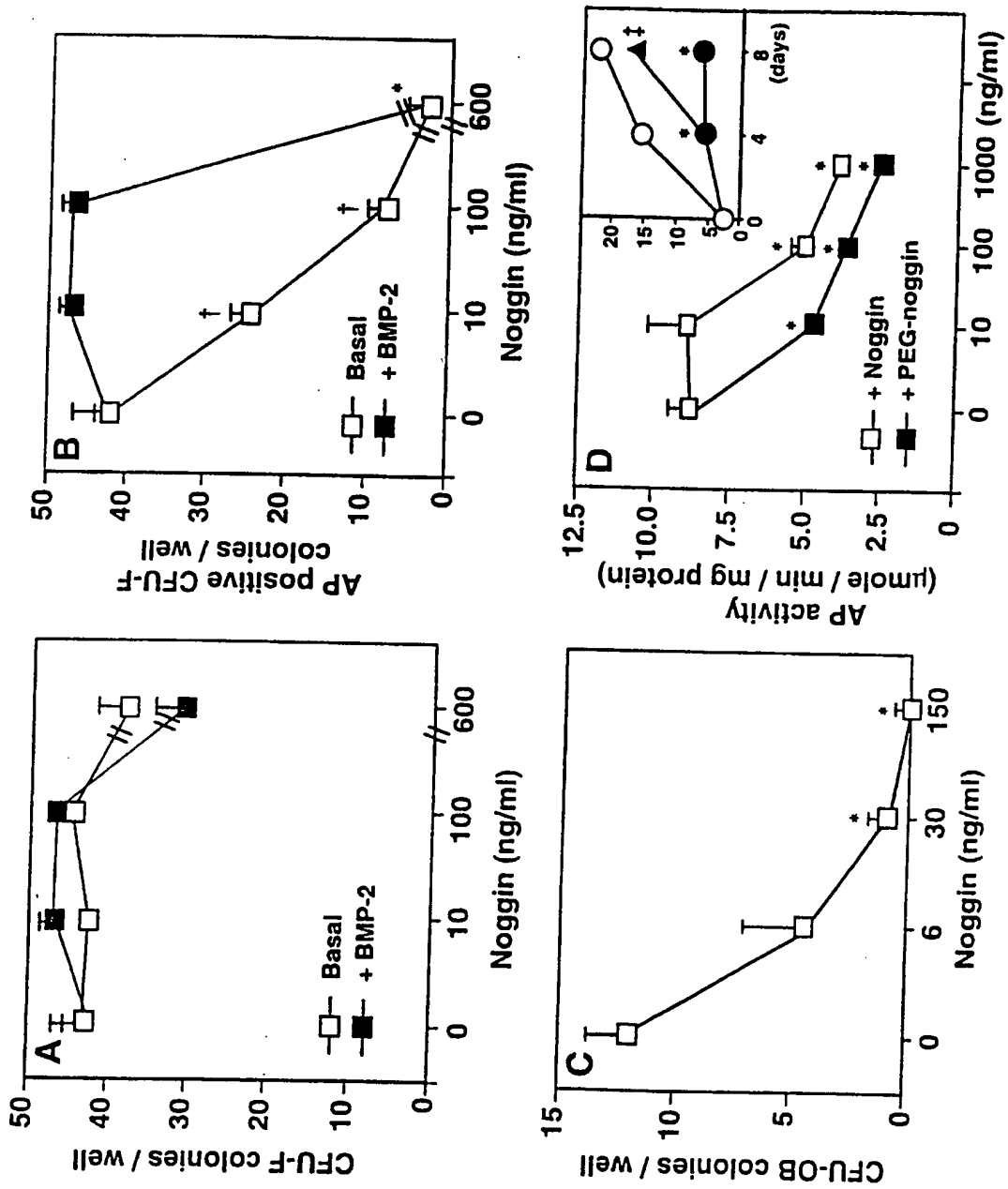
Figure 2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C
CFU-OB colonies / well
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+ PEG-noggin
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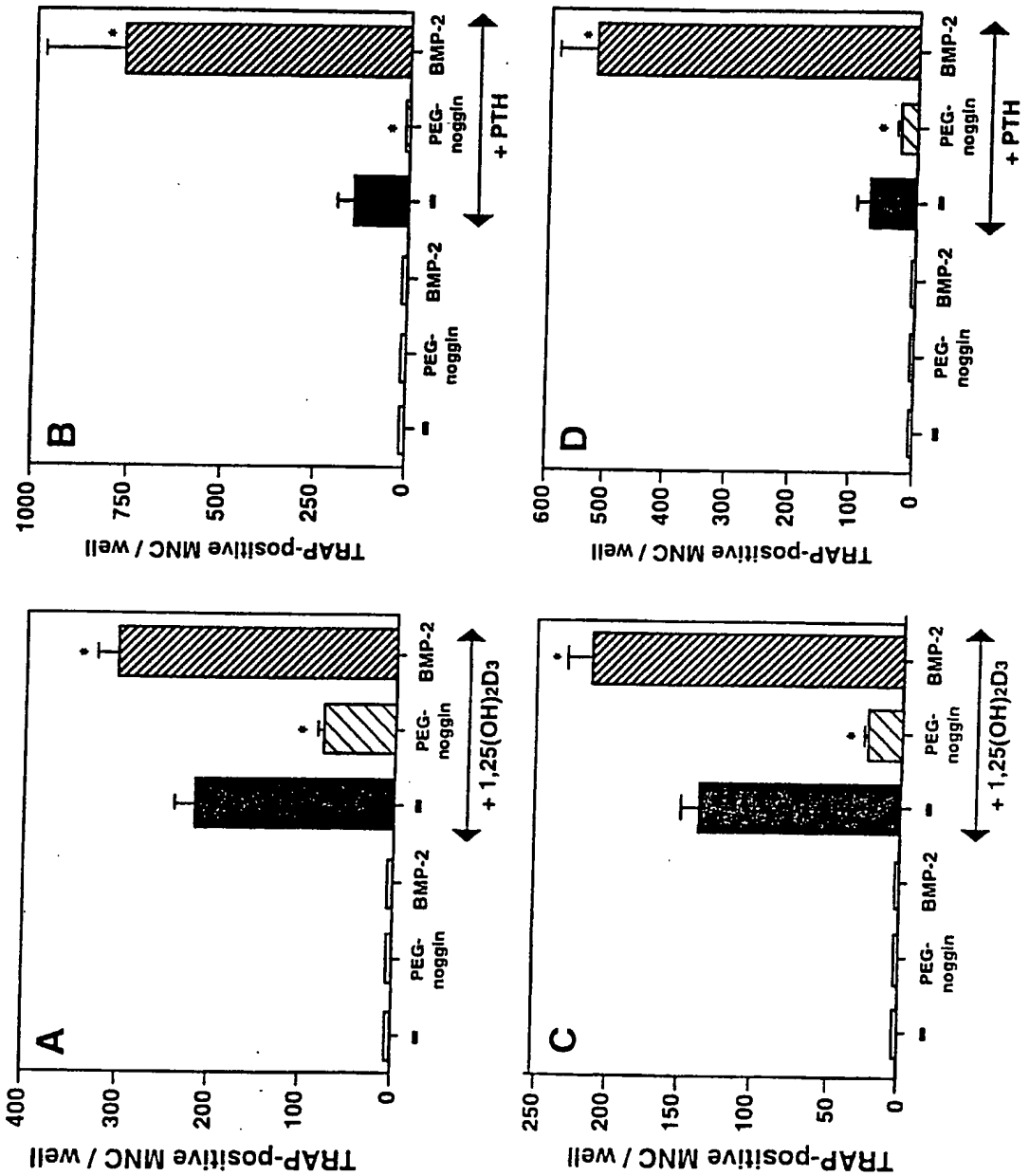
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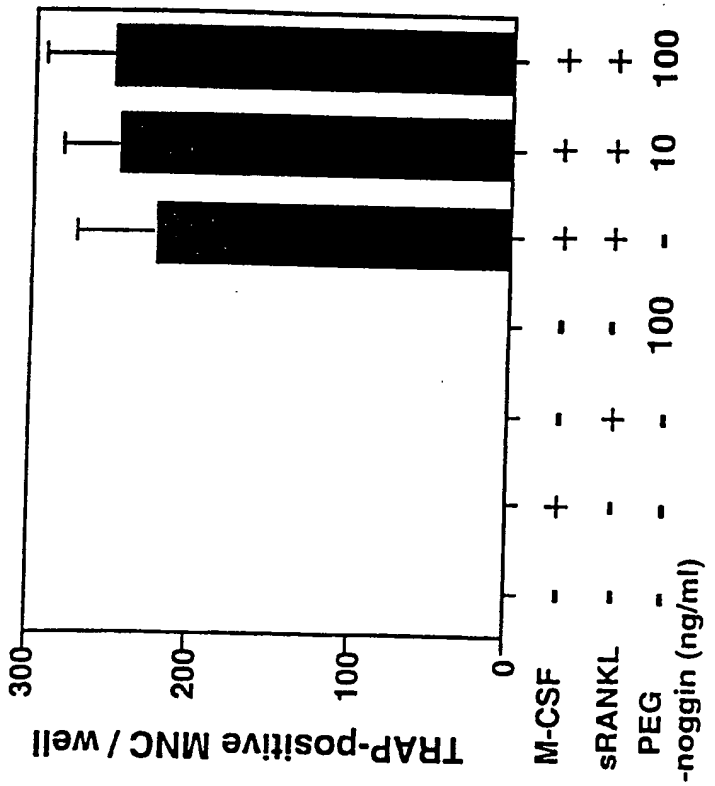
+ 1,25(OH)2D3
+ PTH

Figure 4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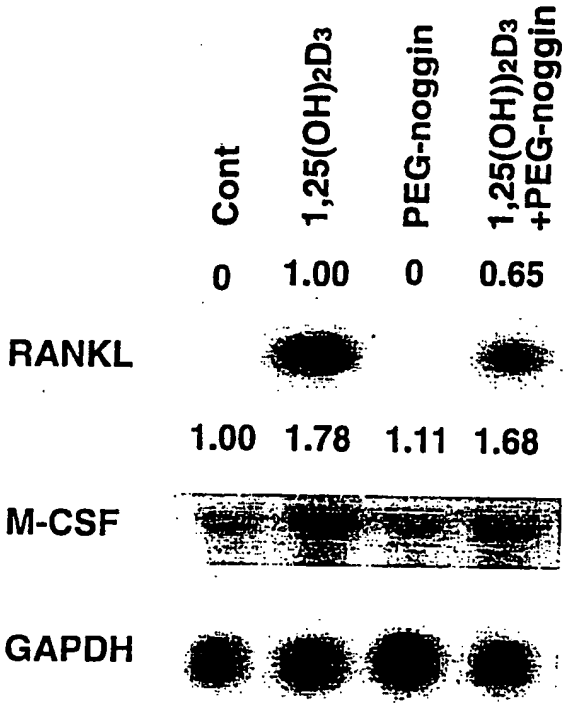
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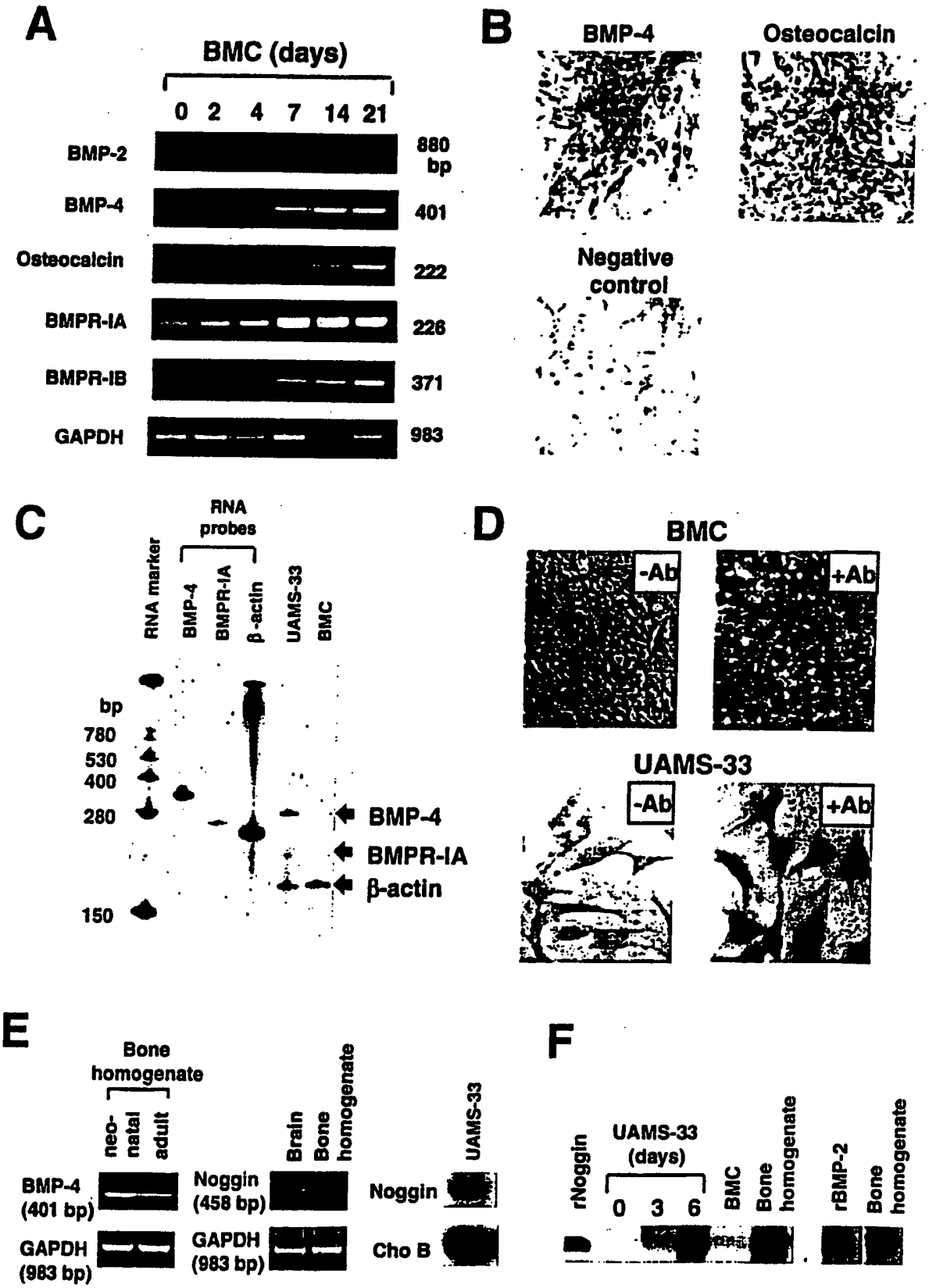
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B
BMP-4
Osteocalcin
Negative control
C
RNA probes
RNA marker
BMP-4
BMPR-IA
β-actin
UAMS-33
BMC
bp
780
530
400
280
150
BMP-4
BMPR-IA
β-actin
D
BMC
-Ab
+Ab
UAMS-33
-Ab
+Ab
E
Bone homogenate
neo-natal
adult
Brain
Bone homogenate
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Noggin (458 bp)
Noggin
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<400> 12
acttggatgg cttacacacc atgc 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12001

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 38/16; C12P 21/06; C12N 5/06, 5/10
US CL :514/02, 12; 435/69.1; 365.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/02, 12; 435/69.1; 365.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN ON LINE: noggin, chordin, cerberus, bone morphogenic protein, BMP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,679,783 A (DE ROBERTIS et al.) 21 October 1997, see abstract.	1-4
Y	DATABASE CAPLUS. DN 127:275374. WILSON, P. et al. 'Concentration-dependent patterning of Xenopus exoderm by BMP4 and its signal transducer Smad1,'abstract, Development, 1997, Vol. 124, No. 16, pages 3177-3184, see abstract.	1-4
X, P	WO 98/49296 A1 (REGENERON PHARMACEUTICALS, INC.) 05 November 1998, see abstract	1-4
A	WO 94/05791 A2 (REGENERON PHARMACEUTICALS, INC.) 17 March 1994, see abstract.	1-4
A	WO 94/05800 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 17 March 1994, see abstract.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
18 AUGUST 1999	18 OCT 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL BORIN
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, species group "A"

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12001

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-4, and drawn to method of suppressing bone resorption.
Group II, claims 5-11, drawn to method of increasing bone density.
Group III, claim 12, drawn to method of regulating action of bone morphogenic peptide.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I, II utilize different products: antagonist of a bone morphogenic peptide in Group I, and an antibody to an antagonist of a bone morphogenic peptide, in Groups II/III. is the technical feature that links Groups I to II. The special technical feature linking Group III with Group I and Group II is noggin and antibody against noggin, respectively. Both noggin and antibody against noggin are known in the art. See, e.g., WO 9405791, WO 9405800. Therefore, the lack of unity is present because the linking technical feature is not a "special technical feature" as defined by PCT Rule 13.2. Inventions I-III independent methods which are not connected in design, operation or effect.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Group A, claims 1-11, drawn to methods of use of noggin or chordin (claims 1-4) or antibodies thereto (claims 5-9).
Group B, claims 1-11, drawn to methods of use of cereberus (claims 1-4) or antibodies thereto (claims 5-9).
Group C, claim 12, drawn to method of use of chordin or antibodies to chordin.

For Groups A and B, noggin and chordin are BMP antagonists, whereas cereberus is an anterior mesoderm protein.

For Group C, chordin and antibodies to chordin are different products having different effect.

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